

Ebola and Marburg Viruses Replicate in Monocyte-Derived Dendritic Cells without Inducing the Production of Cytokines and Full Maturation

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Ebola virus (EBOV) and Marburg virus (MARV) cause rapidly progressive hemorrhagic fever with high mortality and may possess specialized mechanisms to evade immune destruction. We postulated that immune evasion could be due to the ability of EBOV and MARV to interfere with dendritic cells (DCs), which link innate and adaptive immune responses. We demonstrate that EBOV and MARV infected and replicated in primary human DCs without inducing cytokine secretion. Infected DC cultures supported exponential viral growth without releasing interferon (IFN)- α and were impaired in IFN- α production if treated with double-stranded RNA. Moreover, EBOV and MARV impaired the ability of DCs to support T cell proliferation, and infected, immature DCs underwent an anomalous maturation. These findings may explain the profound virulence of EBOV and MARV—DCs are disabled, and an effective early host response is delayed by the necessary reliance on less-efficient secondary mechanisms.

An effective immune response is an animal's last and best defense against an invading pathogen. Thus, the most virulent of organisms in unvaccinated individuals usually outpace and/or subvert in some way the innate and adaptive immune responses. Despite their relative simplicity, viruses have developed multiple strategies to evade detection by and activation of host immune responses. Cytomegalovirus (CMV) interferes with normal antigen presentation in infected cells by blocking peptide transporters [1]. Vaccinia virus (VV) stops the maturation of immature dendritic cells (DCs) into the mature DCs required for efficient antigen presentation [2]. Re-

cently, several viruses, including influenza, and, more recently, other viruses unrelated to the filovirus family have been found to produce an interferon (IFN) antagonist [3].

Ebola virus (EBOV) and Marburg virus (MARV), members of the family *Filoviridae*, cause severe and often fatal hemorrhagic fevers. After infection, humans and nonhuman primates develop severe, rapidly progressing illnesses characterized by fever, hemorrhage in multiple organs, and shock. In sizeable outbreaks, human mortality rates have reached ~90% with EBOV and ~70% with MARV [4–6]. Along with generalized immunosuppression, specific immune failures, such as the delay of antibody responses in lethally infected, compared with surviving, individuals, have been reported [5].

The importance of both effective innate and adaptive immune responses after filovirus infections has been further underscored by recent experiments in SCID, IFN receptor knockout, and STAT-1 knockout mice, which emphasized the importance of both adaptive and innate immunity in the control of filovirus infections [7]. Additional studies have addressed filovirus interactions with specific host cells. It has been demonstrated that human monocytes, macrophages, and endothelial cells are per-

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missive for the exponential growth of both viruses. Additionally, growth in these cell types was accompanied by the secretion of significant concentrations of several chemokines and proinflammatory cytokines, including RANTES, monocyte chemotactic protein-1, macrophage inflammatory protein (MIP)-1 α , tumor necrosis factor (TNF)- α , interleukin (IL)-6, IL-8, and growth-related oncogenic- α [6, 8–11]. On the other hand, EBOV inhibited the ability of infected cells to secrete IFN- α , an important immunomodulatory and antiviral cytokine. This suppression was associated with the EBOV protein VP35 [10, 12].

DCs are specialized cell lineages that form a critical link between the innate and adaptive immune responses [13]. After their infection or uptake of antigen, DCs initiate immune responses via the secretion of chemokines and proinflammatory cytokines and the up-regulation of a variety of costimulatory and chemokine receptors. After maturation, they efficiently present antigens and initiate both adaptive and innate immune responses [14]. Additionally, DCs can further guide immune responses to pathogenic organisms, most likely by the release of specific cytokines, toward the activation of Th1 and/or Th2 arm(s) of T cell responses. Thus, considering their central role in the development of immunity, DCs can be especially important targets for pathogens that are thought to evade host immune responses.

In the present article, we demonstrate that primary human DCs are uniquely vulnerable targets for both EBOV and MARV. Despite viral replication in cultured cells, and unlike the results of previous reports of filovirus infections in monocytes and endothelial cells, infected DCs did not secrete detectable concentrations of proinflammatory or immunoregulatory cytokines. Also of importance and previously unknown, we found that filovirus-infected DCs did not undergo conventional maturation and that a functional impairment resulted—that is, both EBOV and MARV interfered with the allogenic T cell stimulatory capabilities of DCs. Finally, IFN- α secretion in response to a second IFN-inducing stimulus (replication-defective alphavirus) was also potentially inhibited by both viruses. From these data and from previous reports of pathogenesis and virus-induced immune dysfunction during acute EBOV and MARV infections, we hypothesize that filovirus infections of DCs play a pivotal role in the outcome of primary disease. Other testable hypotheses about therapeutic approaches and vaccines and suppositions about why some candidates may prove to be more promising than others may be derived from these studies.

MATERIALS AND METHODS

Culture of primary human DCs and adherent monocytes.

DCs were cultured from peripheral blood as described elsewhere [15]. Mononuclear cells were cultured in complete (c) RPMI 1640 supplemented with 2 mmol/L L-glutamine, 10 mmol/L HEPES buffer, and 1% nonessential amino acids

(NEAA; all from Life Technologies). CD14⁺ cells were cultured (1.5×10^6 cells/mL in 6-well culture plates) in cRPMI medium supplemented with 100 ng/mL recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) and 20 ng/mL rhIL-4 (both from R&D Systems). One-third of the medium and 100% of each cytokine were replaced every other day. The resulting differentiated DCs were >97% CD1a⁺ and <1% CD14⁺. DCs were used on the fourth day of culture in all experiments. Samples from 10 different individuals were used in these experiments, with little variation noted between the responses among individuals.

Primary adherent human monocytes were isolated after the enrichment of peripheral blood monocytes (PBMCs) by centrifugation over ficoll-hypaque. Cells were resuspended in cRPMI and allowed to adhere for 1 h at 37°C, after which nonadherent cells were removed. Immediately after the removal of nonadherent cells, adherent monocytes were infected with either EBOV or MARV, as described below.

Infection of primary human DCs and monocytes with EBOV and MARV. All EBOV- and MARV-infected cells were handled under maximum containment in a biosafety level (BSL) 4 laboratory at the US Army Medical Research Institute for Infectious Diseases (USAMRIID). DCs cultured for 4 days, Vero E6, and adherent monocytes were infected at an MOI of 1 with either EBOV-Zaire [16, 17] or MARV-Musoke [18]. In brief, DCs were pelleted, and DC-conditioned medium was removed. DCs were washed once in serum-free RPMI (SFM), followed by suspension in 0.3 mL of virus. Similarly, Vero E6 and adherent monocytes cells, cultured in 24- or 6-well plates, respectively, were washed once in SFM, and 0.1 mL of virus was added to the monolayers. The viruses were allowed to adsorb for 50 min at 37°C in 5% CO₂. Mock-infected DCs and adherent monocytes received 0.3 or 0.1 mL of SFM, respectively. All infections were done in duplicate or triplicate. All virus stocks were tested for endotoxin and were found to be <0.03 IU/1.5 $\times 10^7$ pfu of virus. The unadsorbed viruses were washed away, the DCs were resuspended in conditioned medium, cRPMI was added to monocytes, and Eagle's MEM (EMEM) supplemented with 2 mmol/L L-glutamine, 10 mmol/L HEPES buffer, and NEAA was added to Vero cells. The DCs were distributed at 5×10^5 cells/mL in 24-well culture plates. Alternatively (as noted), DCs were incubated with 5 or 50 μ g of irradiated (10×10^6 rad) of inactivated (i) EBOV or MARV. Where noted, DCs were incubated (for 24 h) with 20 μ g/mL poly I:C (Calbiochem), 10 ng/mL lipopolysaccharide (LPS), or 5×10^5 (MOI, 1) focus-forming units (ffu) of Venezuelan equine encephalitis (VEE) replicon particles (VRP; a gift from Dr. Christopher Bosio, USAMRIID), prepared as described elsewhere [19], or 100 pfu of inactivated VEE virus strain TC-83, prepared as described elsewhere for hantavirus [20]. At the indicated time points, supernatants were removed for the assay

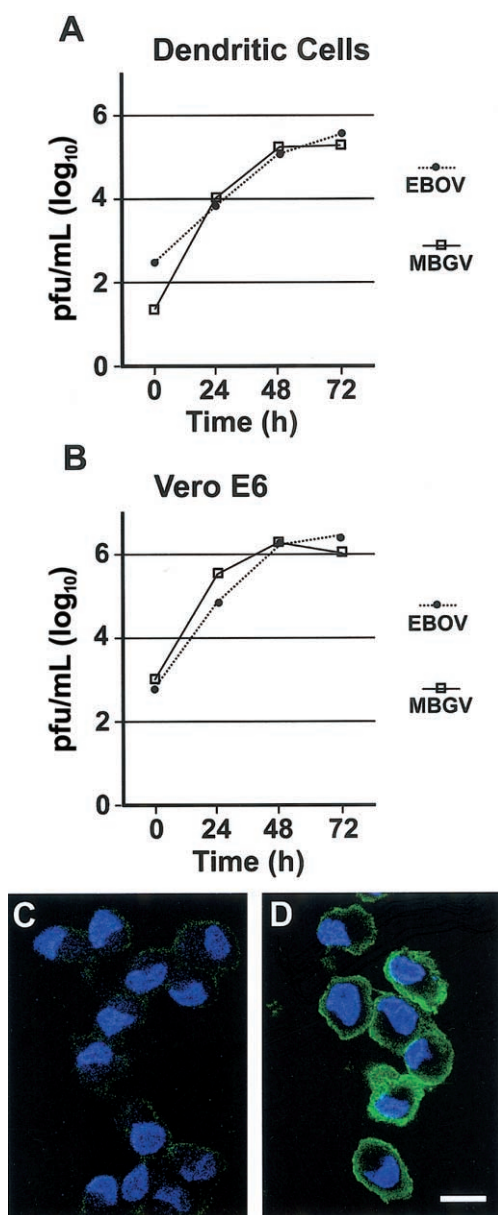


Figure 1. Growth and visualization of Ebola virus (EBOV) and Marburg virus (MARV) in human dendritic cells (DCs). Growth of EBOV and MARV in DCs (A) or Vero E6 cells (B). DCs or Vero E6 cells were infected with EBOV or MARV at an MOI of 1. At the specified time points after infection, cell supernatants were harvested and evaluated for virus load. Data points show the mean no. of EBOV or MARV recovered from supernatants. Uninfected DCs (C) or DCs infected with EBOV at an MOI of 1 (D) were stained for the presence of EBOV proteins. Forty-eight hours after infection, DCs were fixed to spot slides and stained for EBOV proteins, followed by ALEXA 488-conjugated antibodies. DCs were counterstained with 4',6'-diamidini-2-phenylindole hydrochloride, to visualize nuclei, and viewed with a laser confocal microscope. Similar patterns of MARV infection were also observed in DCs. The bar represents 10 μ m in both images. These results are representative of 3 experiments of similar design using samples from 3 different individuals.

of virus titers and cytokine content. Pelleted cells were resuspended in 100 μ L of PBS and used for spot slides, as described below. For experiments where LPS, VRP, or poly I:C were added after infection, cell supernatants were harvested 24 h after the addition of the stimulant (i.e., 48 h after filovirus infection). In some experiments, cells were also harvested and stained for specific cell-surface markers, as described below. Although an MOI of 1 (as measured on Vero E6 cells) suggests that only 63% of DCs would be infected during the first hour, indirect immunofluorescent staining and confocal microscopy showed that virtually all cells contained viral proteins by 48 h. In some instances, DCs were incubated with iEBOV or iMARV, alone or in the presence of 5×10^5 ffu of VRP. Culture supernatants were analyzed for various cytokines or were removed from cultures that contained adherent Vero E6 cells and frozen at -70°C until evaluated for virus load, as described below.

Immunofluorescence. A 20- μ L aliquot of each DC suspension was spotted onto slides with 3-mm glass spots circumscribed by Teflon (Cel-Line Associates). Cells were allowed to air dry, fixed in 10% buffered formalin, frozen at -70°C , and irradiated with 6×10^6 rad before immunostaining. DCs were labeled with either polyclonal guinea pig anti-EBOV or polyclonal guinea pig anti-MARV antibodies [18]. After several washes, the bound antibodies were detected with goat anti-guinea pig antibodies directly labeled with Alexa 488 (Molecular Probes). After incubation, slides were washed several times, mounted with mounting medium for fluorescence (DAKO), and stored at 4°C until they were assessed for viral antigen. Slides were examined by a Radiance2000 multiphoton confocal microscope with LaserSharp2000 Software (Bio-Rad).

Assay of infectious virions. Infectious EBOV and MARV virions were enumerated using a standard plaque assay, as described elsewhere [16, 18]. In brief, culture supernatants were serially diluted 10-fold in cEMEM, and 100 μ L of each dilution was added to wells of Vero E6 cells in duplicate. The viruses were allowed to adsorb for 50 min. After adsorption, $1 \times$ basal medium with Earles salt (EBME; Quality Biological) and 0.5% agarose was added to each well. The plates were incubated for 6 days at 37°C in 5% CO_2 , at which time a second overlay of $1 \times$ EBME/0.5% agarose and 5% neutral red was added to each well. The plates were incubated at 37°C in 5% CO_2 , and plaques were counted 24 h later.

Assay of cytokines in DCs and adherent monocyte culture supernatants. Culture supernatants were assayed for IFN- α , IL-1 β , TNF- α , IL-6, RANTES, MIP-1 α , and IL-10, using Bio-source kits. All ELISAs that tested supernatants containing live virions were done in a BSL-4 laboratory, to avoid known and potential complicating effects of sample irradiation. Assays and controls conformed to the manufacturer's instructions. Results are expressed as the mean \pm SD of triplicate samples.

Allogenic mixed-lymphocyte reaction (MLR). Human T

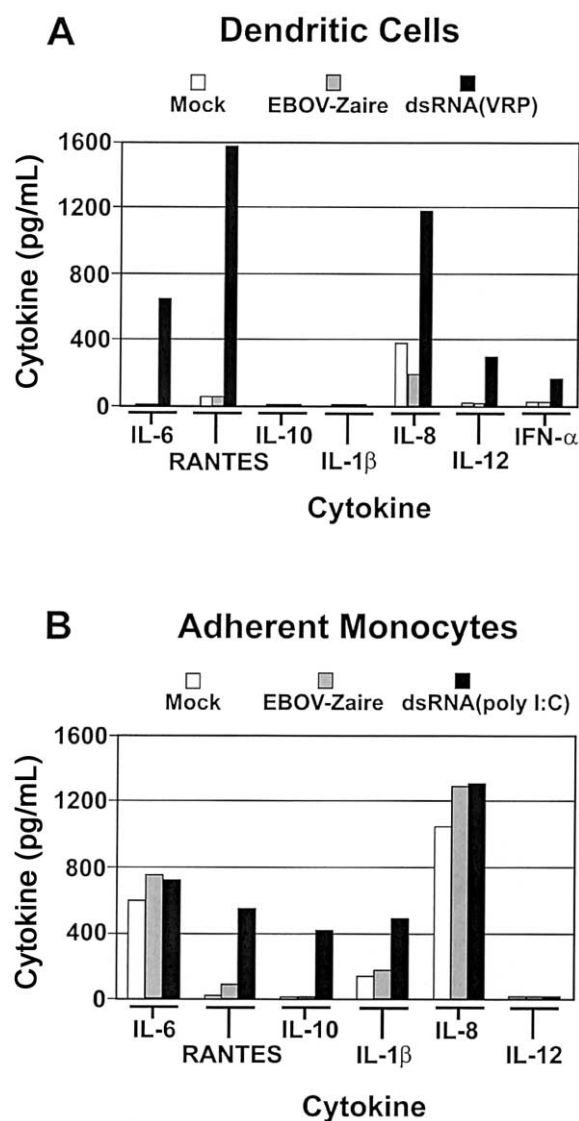


Figure 2. Ebola virus (EBOV) does not induce cytokine secretion from human dendritic cells (DCs). Primary human DCs (A) or adherent monocytes (B) were mock infected (white bars), infected with EBOV-Zaire (gray bars), or exposed to double-stranded (ds) RNA in the form of either poly I:C or virus replicon particles (VRPs) (black bars). Culture supernatants (duplicate samples) were evaluated for the indicated cytokines at 1, 24, 48, and 72 h after infection (48 h is shown here). The SE of the cytokine concentration in each group was <10%. These results are representative of 3 experiments of similar design using samples from 3 different individuals. IFN, interferon; IL, interleukin.

cells were enriched from PBMCs by negative selection. Cells were washed twice in SFM and resuspended in cRPMI that contained heat-inactivated human AB serum (Biowhittaker). Enriched T cells were routinely >95% CD3⁺ and <1% CD19⁺, CD14⁺, and CD56⁺, as determined by flow cytometry. Before coculture with T cells, DCs were washed twice in SFM and resuspended in cRPMI that contained 10% heat-inactivated

human AB serum, 100 ng/mL rhGM-CSF (R&D Systems), and 20 ng/mL rhIL-4 (R&D Systems). T cells (2.5×10^5 cells/well) were cocultured with untreated DCs or DCs treated with 10 μ g of iEBOV, iMARV, or iVV for 4 days in each 200 μ L/well of 96-well plates. iVV was obtained from Vero cells infected with vaccinia strain IHD-J; the cells were subjected to freeze-thaw and mechanical lysis in hypotonic buffer, cellular debris was removed by low-speed spin, and virus was pelleted at 10,000 g through a 34% sucrose cushion. The cells were then pulsed with 1 μ Ci/well of [³H]-thymidine for 18 h, after which the level of [³H]-thymidine incorporation was determined.

Flow cytometry. DC surface markers were analyzed by flow cytometry, as described elsewhere [15]. In brief, the cells were washed in cold PBS/2% fetal bovine serum, and cell-surface staining was done using the following antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-CD80, phycoerythrin (PE)-conjugated anti-CD86, FITC-conjugated anti-HLA-DR, PE-conjugated anti-CD83, FITC-conjugated anti-CD40, PE-conjugated anti-CD11c, PE-conjugated anti-CD1a, FITC-conjugated anti-mouse IgG₁^k, and PE-conjugated anti-mouse IgG2_b^k (all from PharMingen). After incubation with antibodies, cells were washed and fixed with 10% buffered formalin. The cells were analyzed using a FACSCalibur flow cytometer with CellQuest software (Becton Dickinson). A minimum of 10,000 events were collected and analyzed for each sample.

RESULTS

EBOV and MARV readily infect and replicate in human DCs. The permissiveness of PBMC-derived DCs for infection with filoviruses was determined. DCs were infected with either EBOV or MARV, and the kinetics of viral replication determined. EBOV-Zaire and MARV grew exponentially in DCs, with a pattern similar to that observed in Vero E6 cells (figure 1A and 1B). The presence of virus in DCs was confirmed by staining for viral antigens and subsequent analysis by confocal laser microscopy (figure 1C and 1D). At 24 and 48 h, >95% of DCs were viable, whereas, at 72 h after infection, only >60% of DCs appeared to be viable (as assessed by the exclusion of trypan blue).

Filoviruses do not elicit cytokine secretion from DCs. The exposure of DCs to components of bacteria and viruses results in the release of cytokines and chemokines from the affected cell [13, 21]. These mediators play a critical role in the initiation of immune responses. Thus, the immunostimulatory effects of filovirus infection in DCs were assessed by testing culture supernatants from infected and uninfected DCs for various cytokines. Remarkably, at no time (1, 24, 48, or 72 h) was any tested cytokine (IFN- α , IL-1 β , IL-6, IL-10, RANTES, IL-12, or IL-8) detected at concentrations above those of mock controls in culture supernatants of EBOV-Zaire- or MARV-Musoke-infected or uninfected DCs (figure 2A; data not shown). How-

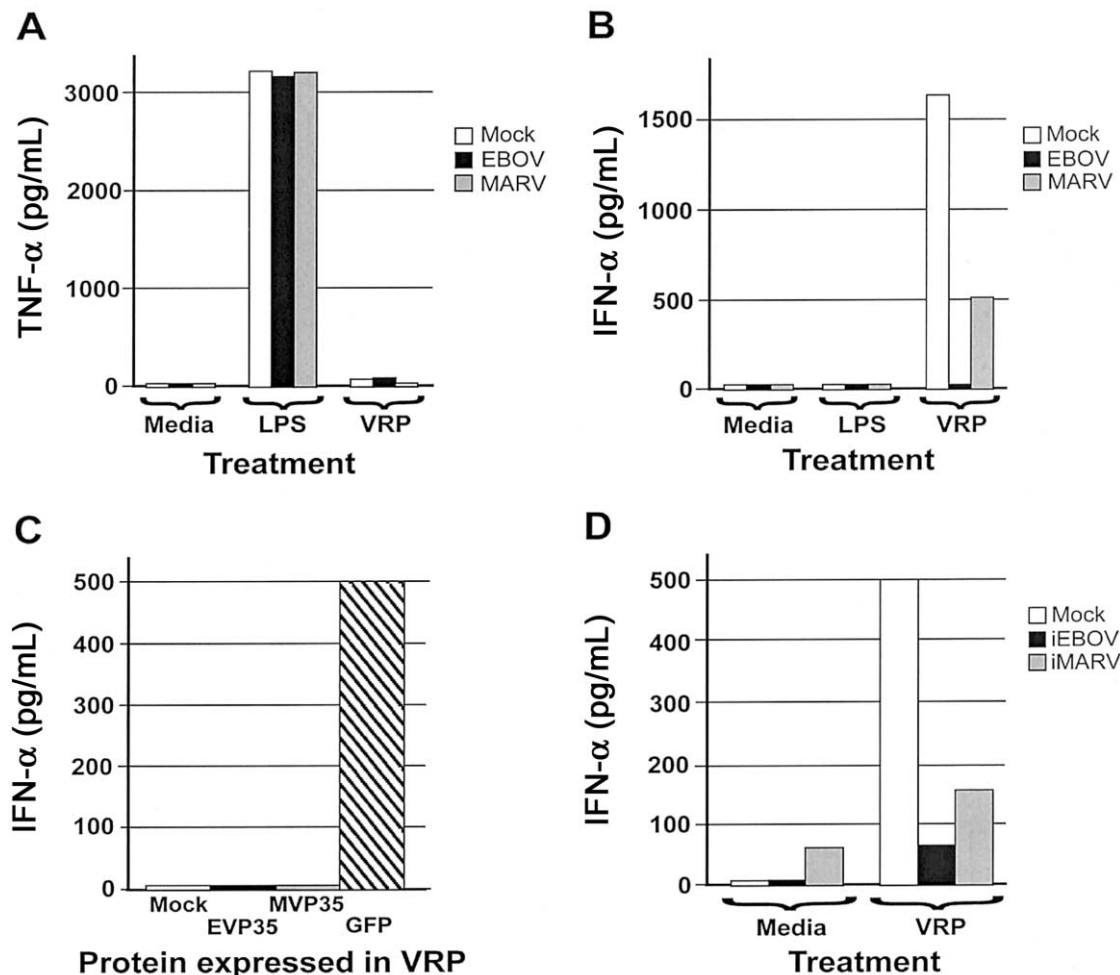


Figure 3. The expression of VP35 down-regulates interferon (IFN)- α induction in human dendritic cells (DCs). Primary human DCs were mock infected (*white bars*) or infected with Ebola virus (EBOV; *black bars*) or Marburg virus (MARV; *gray bars*). Twenty-four hours after infection, the indicated stimulants (virus replicon particles [VRPs] at an MOI of 1 or lipopolysaccharide [LPS], 10 ng/mL) were added, as described in Materials and Methods. Culture supernatants (duplicate samples) were evaluated for tumor necrosis factor (TNF)- α (A) and IFN- α (B) 24 h after the addition of stimulants. C, DCs were infected with VRPs encoding EBOV VP35 (EVP35), MARV VP35 (MVP35), or green fluorescent protein (GFP), and culture supernatants were analyzed for IFN- α 24 h later. D, Inactivated EBOV (iEBOV) (5 μ g/mL) or MARV (iMARV) (5 μ g/mL) was added to cultures of DCs. VRPs (MOI of 1) were added to the indicated cultures in addition to iEBOV or iMARV. Culture supernatants (duplicate samples) were evaluated for IFN- α 24 h later. The SE of the cytokine concentration of a group was <10%. These results are representative of 3 experiments of similar design using samples from 3 different individuals.

ever, adherent monocytes infected with EBOV-Zaire readily secreted IL-1 β , IL-6, RANTES, and IL-8 after infection (figure 2B). We then examined whether EBOV and MARV inhibited the ability of DCs to respond to other activating stimuli. LPS or VRP expressing green fluorescent protein (GFP; as a source of double-stranded [ds] RNA) were added 24 h after DCs had been infected with either MARV or EBOV. Filovirus infection did not appear to have an effect on the ability of DCs to respond to LPS—after the addition of LPS, there were similar concentrations of TNF- α secreted by MARV- or EBOV-infected DCs, compared with uninfected controls (figure 3A). This suggested

that the infected DCs maintained their ability to respond to proinflammatory stimuli. In the absence of filovirus coinfection, VRP potently induced the secretion of IFN- α (figure 3B). However, this IFN response to VRP was completely blocked in EBOV-infected cells, and infection with MARV also inhibited IFN- α secretion substantially (figure 3B). To further examine the mechanism of IFN- α down-regulation, DCs were infected with VRP encoding VP35 (a protein that is part of the nucleocapsid complex) of EBOV or MARV, and the supernatant was tested for IFN- α production after 24 h. The coexpression of either EBOV or MARV VP35 (EVP35 or MVP35, respec-

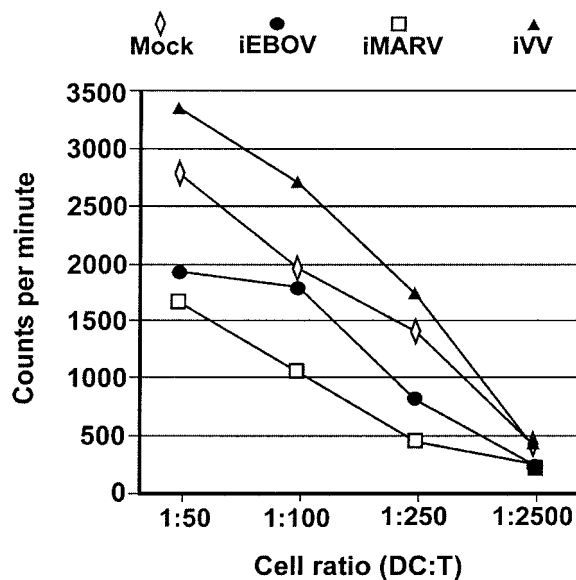


Figure 4. Inactivated Ebola virus (iEBOV) and Marburg virus (iMARV) substantially inhibit allogenetic mixed lymphocyte reactions. Dendritic cells (DCs) treated with iEBOV, iMARV, inactivated vaccinia virus (iVV), or medium alone (Mock) were cultured in varying concentrations (5×10^3 cells/well to 1×10^2 cells/well) with allogenetic T lymphocytes (2.5×10^5 cells/well). On the fourth day of culture, [3 H]-thymidine was added. After an additional 18 h, the incorporation of [3 H]-thymidine by proliferating T cells was assessed. The SE of each experimental group was $<10\%$. These results are representative of 3 experiments of similar design using samples from 3 different individuals.

tively) effectively ablated IFN- α induction by VRP, whereas other coexpressed molecules (GFP, shown here) did not diminish levels of IFN- α (figure 3C). These data show that infection with filoviruses actively suppressed the IFN- α response in DCs and that VP35 could account wholly or in part for this down-regulation.

To determine whether live, replicating virus was required for the inhibition of IFN- α secretion, DCs were exposed to iEBOV or iMARV in the presence or absence of VRP. DCs were treated with VRP for 1 h before the addition of iEBOV or iMARV. In similar experiments described above using live EBOV in DCs, iEBOV did not routinely stimulate the secretion of IFN- α by DCs (figure 3D). Furthermore, the presence of iEBOV and iMARV substantially decreased the amount of IFN- α detected in DCs stimulated with VRP cultures, compared with DCs treated with VRP alone (figure 3D). Unlike iEBOV, the highest concentrations of iMARV stimulated small but detectable amounts of IFN- α from DCs in a dose-dependent manner (data not shown). Of interest, iMARV inhibited IFN- α secretion to an extent similar to that observed with live MARV (figure 3C, 3D). The inhibition of IFN- α secretion was not due to the cytotoxicity of either iEBOV or iMARV in DC cultures, given that $>98\%$ of all cells, regardless of the presence of inactivated

virus, were viable up to 48 h after the addition of iMARV or iEBOV, as determined by the exclusion of trypan blue (data not shown). Together, these data suggest that the replication of these viruses was not required for partial inhibition of IFN- α responses in DCs and that some of the viral proteins (e.g., VP35) may be sufficient for this effect. However, although iEBOV and iMARV were added at higher concentrations (MOIs >100) than live virus in companion experiments, to compensate for the lack of nonstructural proteins likely present among preparations of inactivated virus, compared with those undergoing live replication, it is possible that equivalent amounts of VP35 were not present in DCs at concentrations normally seen during viral infection. Of interest, iMARV, unlike iEBOV, induced modest amounts of IFN- α in DCs (figure 3D). Collectively, these data suggest that EBOV and MARV may differ in their ability to successfully interfere with the secretion of an essential immunoregulatory and antiviral cytokine, IFN- α , in human DCs.

EBOV and MARV inhibit the ability of DCs to support T cell stimulation. Several viruses are known to inhibit the ability of DCs to stimulate T cells as one mechanism of evasion of host immunity [22, 23]. Given the extreme pathogenicity of EBOV and MARV and the apparent inefficiency of immune responses to filoviruses, we hypothesized that these viruses may also apply this strategy to prevent the activation of T cells after the infection of DCs. To address this question, we analyzed the effect of EBOV and MARV on the ability of DCs to support the proliferation of allogenetic T cells. iEBOV and iMARV were added to cultures of allogenetic MLRs that contained DCs and T cells enriched by the peripheral blood of an allogenetic donor. As shown in figure 4, the addition of either EBOV or MARV substantially inhibited the proliferation of T cells, whereas iVV enhanced proliferation, compared with mock-treated DCs. Notably, higher DC:T cell ratios did not overcome the observed inhibition. It has not yet been determined whether live virus has an equivalent or even more profound effect on T cell stimulation than dose-killed virus, as might be anticipated from other results reported here.

EBOV and MARV induce an impaired maturation of DCs. The maturation of DCs is essential for the initiation of the immune response. The increased expression of costimulatory markers, such as major histocompatibility complex (MHC), helps enhance T cell activation, and changes in chemokine receptors are essential for the migration of DCs from affected tissues to local lymph nodes [22, 24]. Thus, the effect of EBOV infection on the expression of various DC maturation markers was analyzed. The infection of DCs with EBOV increased the expression of CD40 and CD80 cells, to levels similar to those observed in DCs treated with LPS (figure 5). However, despite increases in these surface receptors, CD86 and HLA-DR were only slightly increased, whereas CD11c and CD83

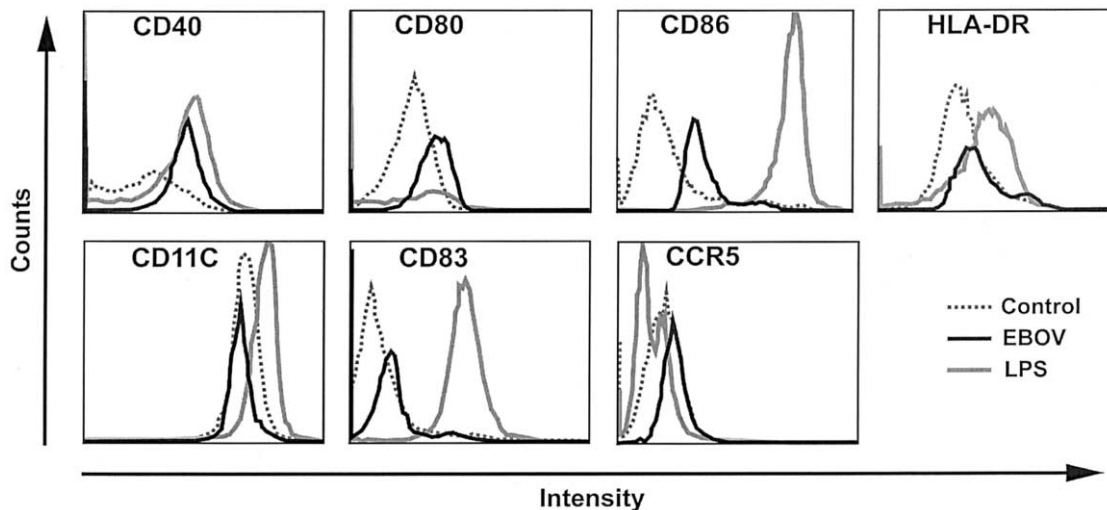


Figure 5. Ebola virus (EBOV) infection induces the anomalous maturation of dendritic cells (DCs). Human DCs were infected with EBOV (*black line*), exposed to lipopolysaccharide (LPS; *gray line*), or mock infected (*dashed line*) for 24 h. The cells were evaluated for expression of CD40, CD80, CD86, HLA-DR, CD11c, CD83, and CCR5 by staining for specific cell-surface markers and analyzed by flow cytometry. These results are representative of 3 experiments of similar design using samples from 3 different individuals.

expression were not augmented at all, as in uninfected DCs (figure 5). Furthermore, CCR5 did not appear to be down-regulated in EBOV-infected DCs (figure 5). We observed similar changes in CD40, CD80, CD86, HLA-DR, CD11c, CD83, and CCR5 when cells were infected with MARV or exposed to either iEBOV or iMARV (data not shown). To determine whether EBOV or MARV virus actively prevented maturation by dsRNA, poly I:C was added 24 h after infection. The expression of cell-surface markers was examined by flow cytometry 24 h after the addition of poly I:C. Despite infection with EBOV and MARV, DCs still underwent maturation in response to poly I:C, as measured by an increase in CD80, CD86, CD40, HLA-DR, HLA-ABC, and CD83 expression similar to that of uninfected controls (data not shown). A similar pattern of expression was observed in infected DCs exposed to LPS as the maturation stimulus (data not shown). Together, these data suggest that EBOV did not stimulate the normal maturation of DCs after infection, and, of importance, EBOV underwent exponential viral replication in the absence of optimal antigen presentation without the efficient triggering of innate immune responses. However, infected DCs were still able to mature in response to poly I:C and LPS, which suggests that the impaired maturation that follows infections with EBOV and MARV may be restored to some level of normality, given the proper stimulus and timing.

DISCUSSION

DCs have been identified as being cells capable of directing both innate and adaptive immune responses [14]. Given their

importance in defense against infectious diseases, they are attractive targets for a variety of pathogens, including viruses. In the present article, we demonstrate that DCs are targets for both EBOV and MARV. Additionally, we show that, although both viruses grew exponentially in DCs, neither stimulated the secretion of a variety of cytokines from DCs, and both actively inhibited the ability of DCs to secrete IFN- α . Furthermore, both iEBOV and iMARV inhibited the T cell stimulatory capacity of DCs.

Recently, a number of reports have described the replication of a variety of viruses in DCs. Although some viruses (e.g., hantavirus, dengue fever virus, and influenza) induce phenotypic maturation—the secretion of cytokines without the interference of T cell proliferation—other viruses have mechanisms to suppress these responses in DCs. For example, measles virus (MV) induces the phenotypic maturation of DCs but suppresses antigen presentation, an event that is associated with the expression of MV glycoproteins on the surface of the cell [25]. On the other hand, herpes simplex virus (HSV) and CMV down-regulate the expression of cell-surface markers, which results in the poor support of T cell proliferation [22, 25, 26]. Our data demonstrate that EBOV and MARV may interfere with the immune response in a manner similar to that of HSV and CMV. As we describe, EBOV does not induce the optimal maturation of DCs; rather, EBOV infection results in small increases in CD86 and HLA-DR, no increase in CD83, and no down-regulation of CCR5 on DC surfaces. Recent studies have demonstrated that the expression of 2 chemokine receptors are also indicators of DC maturation. As DCs mature, they down-reg-

ulate CCR5 while increasing the expression of CCR7. This coordinated regulation of chemokine receptors is presumed to enhance their ability to migrate from the tissue to resident lymph nodes [24].

In addition to the induction of limited DC maturation, iEBOV and iMARV substantially interfered with the ability of DCs to support the proliferation of allogenic T cells. These data suggest that EBOV and MARV may efficiently replicate in DCs without alerting other cells in the immune system, specifically T cells. We recently showed that filoviruses use lipid rafts during entry and egress from cells [27]. These cholesterol-enriched microdomains are known to be critical for variety of physiological functions, including cell-to-cell communication and signal transduction [28, 29]. Therefore, one explanation for the functional perturbations in DCs is that the assembly and release of filovirus virions through lipid-raft microdomains may permanently disorganize the composition and morphology of the rafts, resulting in suboptimal signal transduction and cell-to-cell communication and, ultimately, in the impairment of the ability of DCs to present and process antigens.

IFN- α is a cytokine that is crucial for effective innate immunity against a variety of pathogens and the subsequent regulation of specific immunity [30, 31]. Many viruses have developed mechanisms to interfere with normal IFN activation in the host. As has been demonstrated in primary human macrophages and monocytes, HSV-1 protein ICP34.5 redirects phosphatase to dephosphorylate eukaryotic initiation factor-2, thus interrupting IFN- α activation and enabling continued protein synthesis, despite the presence of activated dsRNA-dependent protein kinase [32]. Other viruses have much more generalized targets of the IFN- α signaling pathway in these cell types. Human CMV specifically inhibits IFN- α -stimulated MHC class I, IFN- α regulatory factor-1, MxA, and 2',5'-oligoadenylate synthetase gene expression and transcriptional activation [33]. Sendai virus produces 2 proteins, C and V, which target separate points of the IFN- α pathway, counteracting the establishment of antiviral states in uninfected cells and preventing signaling from IFN both type I and II receptors, respectively [34].

There is evidence that filoviruses use similar mechanisms for disrupting IFN- α induction, as described above. First, live EBOV infections in endothelial cells interfere with the induction of IFN- α during the early stages of the IFN signaling pathway, most likely during the activation of Jak and STAT molecules [10]. These results were further supported by the observation that the ectopic expression of EBOV VP35 protein substitutes for the influenza IFN- α antagonist NS1 in its capacity to block IFN- α production in cells infected with an NS1 knockout variant of influenza virus [12]. A later study confirmed that, in addition to endothelial cells, macrophages in-

fects with live EBOV do not secrete IFN- α after infection and are unable to do so after additional exposure to dsRNA [9]. Our data clearly demonstrate that DCs infected with EBOV were similarly unresponsive to further stimulation with viral products, as illustrated by the absence of IFN- α in culture supernatants, which further supports the observation that EBOV effectively interferes with the secretion of IFN- α from target cells. We observed that the inhibition of IFN- α secretion by DCs did not depend on replicating EBOV, which suggests that irradiated virus may contain sufficient structural protein VP35 to account for this effect, a finding that may be important for vaccination considerations. In addition, the ability of inactivated virus to disrupt the induction of IFN- α responses suggests the possibility that noninfectious virus particles in the infected host may exert immunomodulating effects on bystander cells, contributing to the immunosuppression observed in the infected host. Of interest, DCs infected with MARV were able to secrete small amounts of IFN- α after additional exposure to a source of dsRNA, and iMARV also stimulated the secretion of small amounts of IFN- α . However, EBOV completely inhibited this pathway. It was unclear whether iMARV directly interfered with VPR-mediated IFN- α secretion in cells that had taken up inactivated virus, because only slightly greater concentrations of IFN- α were detected in DC cultures that contained either iMARV alone, compared with those in which VRPs were present. This suggests that the mechanisms or target-binding specificities used by EBOV and MARV to manipulate this arm of innate immunity in host cells may not be identical, which would not be surprising, given that the VP35 IFN- α antagonists of these viruses share only ~35% identity [35].

In the present article, we have clearly demonstrated that, despite the exponential growth of filoviruses in DCs, the infected cells are impaired in several arms of immune defense, including the induction of cytokine secretion, responsiveness to further stimulation, impaired maturation, and support of T cell proliferation. Although the results of previous studies have elucidated a clear role for monocytes and macrophages in defense against filovirus infection via their ability to secrete a wide array of cytokines and chemokines after infection, those experiments do not explain the immunosuppression observed during early stages of filovirus-mediated disease. In their totality, our findings may explain the profound virulence of EBOV and MARV, given that DCs (the key sentinels of innate and adaptive immune responses) are disabled, which allows EBOV and MARV to grow undetected until the numbers of virus are beyond the ability of the host immune response to contain the infection. With further understanding of specific host pathways manipulated by EBOV and MARV, we can begin to develop effective therapeutics and treatments for filovirus-mediated disease.

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References

1. Tortorella D, Gewurz BE, Furman MH, Schust DJ, Ploegh HL. Viral subversion of the immune system. *Annu Rev Immunol* **2000**; 18:861–926.
2. Engelmayer J, Larsson M, Subklewe M, et al. Vaccinia virus inhibits the maturation of human dendritic cells: a novel mechanism of immune evasion. *J Immunol* **1999**; 163:6762–8.
3. Flint S, Enquist L, Krug R, Racaniello V, Skalka A. Virus offense meets host defense: principles of virology: molecular biology, pathogenesis and control. Washington, DC: ASM Press, **2000**:488.
4. Bertherat E, Talarmin A, Zeller H. Democratic Republic of Congo: between civil war and the Marburg virus. *Int Comm Tech Sci Coord Durba Epidemiol* **1999**; 59:201–4.
5. Feldmann H, Klenk HD. Marburg and Ebola viruses. *Adv Virus Res* **1996**; 47:1–52.
6. Feldmann H, Bugany H, Mahner F, Klenk HD, Drenckhahn D, Schnittler HJ. Filovirus-induced endothelial leakage triggered by infected monocytes/macrophages. *J Virol* **1996**; 70:2208–14.
7. Bray M. The role of the type I interferon response in the resistance of mice to filovirus infection. *J Gen Virol* **2001**; 82:1365–73.
8. Hensley LE, Young HA, Jahrling PB, Geisbert TW. Proinflammatory response during Ebola virus infection of primate models: possible involvement of the tumor necrosis factor receptor superfamily. *Immunol Lett* **2002**; 80:169–79.
9. Gupta M, Manhanty S, Ahmed R, Rollin P. Monocyte derived human macrophages and peripheral blood mononuclear cells infected with Ebola virus secrete MIP-1 alpha and TNF-alpha and inhibit poly-IC induced IFN-alpha in vitro. *Virology* **2001**; 284:20–5.
10. Harcourt BH, Sanchez A, Offermann MK. Ebola virus selectively inhibits responses to interferons, but not to interleukin-1beta, in endothelial cells. *J Virol* **1999**; 73:3491–6.
11. Stroher U, West E, Bugany H, Klenk HD, Schnittler HJ, Feldmann H. Infection and activation of monocytes by Marburg and Ebola viruses. *J Virol* **2001**; 75:11025–33.
12. Basler CE, Wang X, Muhlberger E, et al. The Ebola virus VP35 protein functions as a type I IFN antagonist. *Proc Natl Acad Sci USA* **2000**; 97:12289–94.
13. Liu YJ. Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. *Cell* **2001**; 106:259–62.
14. Steinman RM. Dendritic cells and the control of immunity: enhancing the efficiency of antigen presentation. *Mt Sinai J Med* **2001**; 68:106–66.
15. Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med* **1994**; 179:1109–18.
16. Jahrling PB, Geisbert J, Swarengen JR, et al. Passive immunization of Ebola virus-infected cynomolgus monkeys with immunoglobulin from hyperimmune horses. *Arch Virol Suppl* **1996**; 11:135–40.
17. Jahrling PB, Geisbert TW, Jaax NK, Hanes MA, Ksiazek TG, Peters CJ. Experimental infection of cynomolgus macaques with Ebola-Reston filoviruses from the 1989–1990 U.S. epizootic. *Arch Virol Suppl* **1996**; 11:115–34.
18. Hevey M, Negley D, Pushko P, Smith J, Schmaljohn A. Marburg virus vaccines based upon alphavirus replicons protect guinea pigs and non-human primates. *Virology* **1998**; 251:28–37.
19. Pushko P, Bray M, Ludwig GV, et al. Recombinant RNA replicons derived from attenuated Venezuelan equine encephalitis virus protect guinea pigs and mice from Ebola hemorrhagic fever virus. *Vaccine* **2000**; 19:142–53.
20. Schmaljohn AL, Li D, Negley DL, et al. Isolation and initial characterization of a newfound hantavirus from California. *Virology* **1995**; 206:963–72.
21. Cella M, Salio M, Sakakibara Y, Langen H, Julkunen I, Lanzavecchia A. Maturation, activation, and protection of dendritic cells induced by double-stranded RNA. *J Exp Med* **1999**; 189:821–9.
22. Karp CL. Measles: immunosuppression, interleukin-12, and complement receptors. *Immunol Rev* **1999**; 168:91–101.
23. Salio M, Cella M, Suter M, Lanzavecchia A. Inhibition of dendritic cell maturation by herpes simplex virus. *Eur J Immunol* **1999**; 29:3245–53.
24. Jarrossay D, Napolitani G, Colonna M, Sallusto F, Lanzavecchia A. Specialization and complementarity in microbial molecule recognition by human myeloid and plasmacytoid dendritic cells. *Eur J Immunol* **2001**; 31:3388–93.
25. Klagge IM, Schneider-Schaulies S. Virus interactions with dendritic cells. *J Gen Virol* **1999**; 80:823–33.
26. Kruse M, Rosorius O, Kratzer F, et al. Mature dendritic cells infected with herpes simplex virus type 1 exhibit inhibited T-cell stimulatory capacity. *J Virol* **2000**; 74:7127–36.
27. Bavari S, Bosio CM, Wiegand E, et al. Lipid raft microdomains: a gateway for compartmentalized trafficking of Ebola and Marburg viruses. *J Exp Med* **2002**; 195:593–602.
28. Machy P, Serre K, Baillet M, Leserman L. Induction of MHC class I presentation of exogenous antigen by dendritic cells is controlled by CD4⁺ T cells engaging class II molecules in cholesterol-rich domains. *J Immunol* **2002**; 168:1172–80.
29. Vidalain PO, Azocar O, Lamouille B, Astier A, Rabourdin-Combe C, Servet-Delprat C. Measles virus induces functional TRAIL production by human dendritic cells. *J Virol* **2000**; 74:556–9.
30. Belardelli F. Role of interferons and other cytokines in the regulation of the immune response. *Apmis* **1995**; 103:161–79.
31. Bogdan C. The function of type I interferons in antimicrobial immunity. *Curr Opin Immunol* **2000**; 12:419–24.
32. Cassady KA, Gross M, Roizman B. The second-site mutation in the herpes simplex virus recombinants lacking the gamma134.5 genes precludes shutoff of protein synthesis by blocking the phosphorylation of eIF-2alpha. *J Virol* **1998**; 72:7005–11.
33. Mocarski ES Jr. Immunomodulation by cytomegaloviruses: manipulative strategies beyond evasion. *Trends Microbiol* **2002**; 10:332–9.
34. Komatsu T, Takeuchi K, Yokoo J, Tanaka Y, Gotoh B. Sendai virus blocks alpha interferon signaling to signal transducers and activators of transcription. *J Virol* **2000**; 74:2477–80.
35. Bukreyev AA, Volchkov VE, Blinov VM, Netesov SV. The VP35 and VP40 proteins of filoviruses: homology between Marburg and Ebola viruses. *FEBS Lett* **1993**; 322:41–6.

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